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Cdk1 interplays with Oct4 to repress differentiation of embryonic stem cells into trophectoderm

Lei Li [1], Juanjuan Wang [1], Jie Hou [1], Zhaojia Wu [1], Yuan Zhuang, Mengxue Lu, Yiran Zhang, Xiaohan Zhou, Zhaoyan Li, Wei Xiao *, Weiwei Zhang *

College of Life Sciences, Capital Normal University, Beijing, China

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ABSTRACT

Cdk1 plays an important role in undifferentiated ES cells, but the underlying mechanism remains unclear. This study explores how Cdk1 collaborates with Oct4 to inhibit differentiation in mouse ES cells. We show a direct interaction between Cdk1 and Oct4, whereas other Cdk members, including Cdk2 and Cdk4, fail to associate with Oct4. By immunocytochemistry we show that Cdk1 and Oct4 co-localize in ES cells. The biological function of the Cdk1–Oct4 complex was also addressed. We found that Cdk1 enhances the binding of Oct4 on the trophectoderm marker *Cdx2* and promotes *Cdx2* repression. This regulation is independent of Cyclins and of the kinase activity of Cdk1. Our study explains how Cdk1 and Oct4 interplay to inhibit ES cell differentiation into trophectoderm and thereby maintain stemness.

Structured summary of protein interactions:
Cdk1 physically interacts with **Oct4** by anti tag coimmunoprecipitation (View interaction)
Oct4 binds to **SOX-2** by pull down (View interaction)
Cdk1 physically interacts with **Oct4** and **cyclin-B1** by anti bait coimmunoprecipitation (View interaction)
Oct4 binds to **Cdk1** by pull down (View interaction)
Cdk1 and **Oct4** colocalize by fluorescence microscopy (View interaction)
Oct4 physically interacts with **Sox2** by anti bait coimmunoprecipitation (View interaction)

1. Introduction

Embryonic stem (ES) cells, which can be derived from the inner cell mass (ICM) of early blastocysts [1], are pluripotent and capable of self-renewal [2]. The embryonic origin allows ES cells to serve as a good model for studying the early embryogenesis. On the other hand, the unique properties of ES cells decide their potential application in cell transplantation, tissue engineering and drug development [3–5]. Understanding the molecular mechanisms underlying self-renewal and pluripotency of ES cells is critical to achieve their potentials both in basic research and clinic application.

In recent years, significant progress has been made toward understanding undifferentiation- or differentiation-related factors regulating the characteristics of ES cells, including core transcription factors, such as Oct4 [6], Sox2 [7], and Nanog [8,9]. In vivo, these factors exhibit restricted expression profiles and play essential roles in the process of early embryo development. In ES cells, these regulators are critical in maintaining the stemness. Oct4, also known as Pou5f1, is a key player in maintaining the pluripotent state of self-renewing ES cells. ES cells are particularly sensitive to dosage alterations in Oct4: twofold induction of *Oct4* led to ES cells differentiation into primitive endoderm and mesoderm. Loss of Oct4, on the other hand, triggers the formation of trophectoderm lineages [10]. In vivo, the proper development of the ICM and trophectoderm requires the interplay between Oct4 and caudal-type homeodomain transcription factor Cdx2. Cdx2 is initially co-expressed with Oct4 and they form a complex for the reciprocal repression of their target genes in ES cells. Cdx2 can directly bind to the Oct4 promoter to inhibit its transcription [11]. Elevation of Cdx2 level in ES cells represses the gene activity of *Oct4* and resulted in cell differentiation into trophectoderm [11]. During

* Corresponding authors. Addresses: College of Life Sciences, The Capital Normal University, 105 Xi San Huan Bei Road, Hai Dian District, #732 Lab Building, Beijing 100048, China (W. Xiao); College of Life Sciences, The Capital Normal University, 105 Xi San Huan Bei Road, Hai Dian District, #703 Lab Building, Beijing 100048, China (W. Zhang).

E-mail addresses: weixiao@mail.cnu.edu.cn (W. Xiao), zhangww@mail.cnu.edu.cn, zhangweiwei2002@hotmail.com (W. Zhang).

[1] Contributed equally to this work.

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blastocyst formation, Oct4 expression mainly exist in ICM, while the expression of Cdx2 is highly rich in trophectoderm [12]. Their restricted expression profiles highlight the importance of Oct4 and Cdx2 in regulating early embryonic development.

The ability of mammalian cells to divide is mainly attributed to the presence of Cyclin-dependent kinases (Cdks) and their binding partners, Cyclins [13]. Among the Cdks, Cdk1 is unique due to the observation that Cdk1 alone is sufficient to drive the cell cycle progress [14], which indicates that Cdk1 can compensate other Cdks in cell cycle. Its central role in cell cycle regulation may serve to explain why *Cdk1* deletion leads to early embryonic lethality [14]. Interestingly, several studies reported that Cdk1 is involved in regulating cell differentiation. Ullah et al. showed that Cdk1 can repress the differentiation of trophectoderm stem cells into giant cells [15]. Inhibition of Cdk1 mediated by either specific inhibitor RO3306 or RNA interference leads to apoptosis of the ES cells [15]. Our previous study characterized the *Cdk1*-depleted ES cells and found that Cdk1 is indispensable for the undifferentiated self-renewing state of ES cells. Depletion of *Cdk1* results in decrease of self-renewal genes and increase of differentiation-related genes [16]. However, this study could not distinguish whether its regulation on these genes is direct or indirect. Of note, Wang and his colleagues used affinity purification coupled with mass spectrometry to identify Cdk1 as a member of the Oct4 interactome [17]. Thus, we hypothesized that Cdk1 functions through its physical interaction with Oct4 so as to transcriptionally regulate downstream stemness- or differentiation-related target genes.

In this study, we presented the evidence that Cdk1 is an interaction partner of Oct4. They can form a complex through a direct protein–protein association. By using the immunocytochemistry (ICC) assay, we also detected co-localization of Cdk1 and Oct4 in ES cells. More importantly, their interaction was required for Oct4-mediated repression of *Cdx2* transcription. Interestingly, this role of Cdk1 in ES cells seemed cell-cycle independent. These findings explained the detailed mechanism underlying Cdk1 function in ES cells, and point to a novel role for Cdk1 in transcriptional regulation and differentiation repression.

2. Materials and methods

2.1. Cell culture

Mouse E14 ES cells (ATCC) were cultured under a feeder-free condition at 37 °C with 5% CO_2. The cells were maintained on gelatin-coated dishes in Dulbecco's modified Eagle medium (DMEM; GIBCO), supplemented with 15% heat-inactivated fetal bovine serum (FBS; GIBCO), 0.1 mM β-mercaptoethanol (GIBCO), 2 mM L-glutamine, 0.1 mM MEM non-essential amino acid, 5000 U/ml penicillin/streptomycin and 1000 U/ml of LIF (Chemicon).

2.2. Knockdown plasmids and cell transfection

Oct4 and Cdk1 knockdown plasmid was constructed according to the previous reports [18,19]. Transfection of shRNA oligo was performed using Lipofectamine 2000 (Invitrogen). For knockdown, 4 μg of shRNA plasmids were transfected into ES cells on 35 mm plates, and maintained for 2–6 days prior to RNA or protein harvesting.

2.3. Protein extraction and Western blotting

Total protein was extracted by lysing cells with the whole cell extraction buffer (Tris, 50 mM; Nacl, 150 mM; NP40, 1%; Glycerol, 10%; EDTA, 1 mM; PMSF, 1 mM). Thirty micrograms of the total protein were separated by SDS–PAGE and transferred to PVDF membrane. The membrane was blocked with 5% milk and probed with specific primary antibodies and secondary antibodies. The blots were developed with ECL Advance Western Blotting Detection Kit (Amersham). Anti-Cdk1 antibodies (Bioworld, BS1820; Cell Signaling, Y15; Abcam, E161, Santa Cruz, sc-53219), anti-Oct4 antibody (Santa Cruz, sc-8628), anti-Oct4 antibody (Santa Cruz, sc-8628), anti-HA probe antibody (Santa Cruz, sc-7392), anti-Flag M2 antibody (Sigma, F1804) and mouse anti-β actin antibody (Boster, BM0627) were used.

2.4. Electrophoretic mobility shift assay (EMSA)

Approximately 1 ng of the probe was incubated together with either 100 ng of full length GST-tagged Cdk1 or Oct4 protein or 10–20 μg of cell extracts for 30 min at 25 °C in a final volume of 20 μl. For shift assays, 2 μg of the corresponding antibody were added after 30 min and incubation was continued for 1 h at 4 °C. Subsequently the binding reaction was separated on a 5.5–7% polyacrylamide gel in 1× TB 90 mM Tris, 90 mM boric acid.

2.5. Immunoprecipitation

Five hundred microgram protein samples in a total volume of 500 μl were immunoprecipitated with 2 μg of antibody and 20 μl of Protein-A beads (for rabbit polyclonal antibodies) or Protein-G beads (for mouse monoclonal antibodies). The samples were rotated at 4 °C overnight. The beads were washed 4× with 1 ml of cold NP40 lysis buffer containing protease inhibitors. The beads were then boiled for 10 min in the presence of 25 μl 2× sample buffer and the released proteins fractionated by SDS–PAGE in 12% or 15% gels. Proteins were detected by immunoblotting as described above.

2.6. GST pull-down assay

Purified GST-fusion protein were precleared with GST beads (GE Healthcare, 17-0756-0), for 1 h and incubated with GST or His-tagged Oct4 fusion proteins overnight at 4 °C. Protein-bound GST beads were washed 4× with lysis buffer and eluted in SDS–PAGE sample buffer. Eluted proteins were analyzed by immunoblotting.

2.7. Luciferase reporter assay

Cells were seeded in 24-well plates at a density of 1×10^5. After 24 h, the cells were transfected using Lipofectamine 2000 (Invitrogen). Briefly, luciferase reporter constructs (400 ng), pcDNA–Cdk1 or pcDNA–Oct4 plasmids (400 ng) and the pRL-SV40 Renilla luciferase construct (5 ng) were co-transfected into the wells. Cell extracts were prepared 48 h after transfection and the luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega).

2.8. Immunocytochemistry

ES Cells were fixed with 4% formaldehyde for 30 min, washed 4× over 30 min with PBS + 0.25 g Tween 20 (PBST) and blocked with 5% horse serum in PBST. Primary mouse anti-Cdk1 (Santa-Cruz, SC-53219, 1:250) and goat anti-Oct4 (SantaCruz, SC-8628 1:250) antibodies were applied in blocking solution for 1 h. After washing in PBST, coverslips were incubated with Alexa546-conjugated anti-mouse (Molecular Probes, 1:3000) and Alexa488-conjugated anti goat (Molecular Probes, 1:2000) secondary antibodies for 20 min in the presence of DAPI (2 μg/ml) for 20 min before washing again with PBST and mounting. Images were captured with a 20× oil emersion objective lens, and all red and all green images were adjusted identically in order to generate the merge images.

2.9. Chromatin immunoprecipitation

Cdk1-depleted and mock-transfected ES cells were cross-linked with 1% formaldehyde for 10 min at room temperature and formaldehyde was then inactivated by the addition of 125 mM glycine. Chromatin extracts containing DNA fragments with an average size of 500 bp were immunoprecipitated using anti-Oct4 (sc-8628, Santa Cruz). PCR analyses were performed for immunoprecipitated DNA.

3. Result

3.1. Both Oct4 and Cdk1 were highly expressed in undifferentiated es cells

If Cdk1 functions together with Oct4 through protein–protein interaction to maintain the undifferentiated state of ES cells, their expression levels should exhibit a close correlation along with cell undifferentiation. Thus, we induced ES cells to differentiate by retinoic acid (RA) treatment or LIF withdrawal from the culture medium. Results showed that similar with Oct4, both RA induction and LIF removal led to reduction in the expression level of Cdk1 (detected by antibody BS1820) (Fig. 1). Since there are different modification molecules of Cdk1 including its enzymatic active form (dephosphorylated at the site of tyrosine 15) and inactive form (phosphorylated at the site of tyrosine 15), we also profiled the expression level of these molecules. Results showed that the decrease of both Oct4 and Cdk1 (whole; active form; inactive form) was more obvious upon RA treatment than LIF withdrawal. Since these two assays drive ES cells to differentiate into different lineages, we could conclude that Oct4 and Cdk1 preferably expressed in pluripotent ES cells, and exhibited similar expression profile along differentiation.

3.2. Cdk1 can directly interact with Oct4

Although Wang et al. showed an existence of Cdk1 in the Oct4 interactome, a more recent study by Pardo et al. did not observe Cdk1 in the Oct4 complex [20]. Thus, we were interested to confirm whether these two proteins can interact. Firstly, ES cells were transfected with expression plasmids inserted with the open reading frame of Cdk1 which was fused with HA-tag at the carboxyl terminus. After 2-day antibiotic selection, the cell lysates were subjected to immune-precipitation with anti-HA monoclonal antibody, followed by Western blotting with anti-Oct4 antibody. Results showed that HA-tagged Cdk1 was successfully overexpressed, while the expression level of Oct4 did not change (Fig. 2A, lower panel). This observation allowed us to further perform immune-precipitation assay. As shown in Fig. 2A, we observed complex formation between Cdk1 and Oct4 protein. To examine whether this complex formation with Oct4 is related with the kinase activity of Cdk1, we inserted the open reading frame expressing kinase-dead Cdk1 mutant (D146 N) into the same expression vector [21]. Interestingly, we also detected its association with Oct4 (Fig 2A), indicating that the interaction between Cdk1 and Oct4 was independent of Cdk1 kinase activity. To further confirm the interaction between endogenous Cdk1 and Oct4, the co-immunoprecipitation assay was carried out by using the lysates of wild type ES cells, for affinity capture with anti-Cdk1 antibody and Western blotting with anti-Oct4 antibody. The Cdk1 IP elution sample was qualified by the existence of Cyclin A and B1 (Fig. 2B). As a result, a strong band of Oct4 was detected, showing that Oct4 was co-purified with Cdk1 (Fig. 2B).

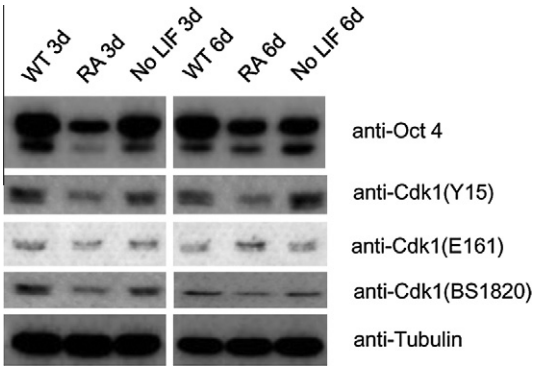

Fig. 1. Undifferentiated mouse ES cells expressed high levels of Cdk1 and Oct4. ES cells were treated with 1 μM RA or cultured in LIF-withdrawn medium for 3 and 6 days, respectively. The expression levels of Cdk1 and Oct4 were monitored by Western blot.

The above studies only confirmed that Cdk1 could be found in an Oct4 complex. In order to determine whether Cdk1 can directly associate with Oct4, pull-down assays with Ni–NTA agrose beads by using purified Cdk1 and Oct4 Protein were performed. Bacterially expressed His–Oct4 fusion protein was captured by Ni–NTA beads and incubated with purified GST–Cdk1 fusion protein. GST-Sox2 fusion protein was used as a positive control, since Sox2 is a well-established Oct4 partner [22]. Interaction between Oct4 and Sox2 was observed (Fig. 2C, right panel). Importantly, GST-tagged Cdk1 was successfully pulled down by His-tagged Oct4, but not the GST mock protein (Fig. 2C, left panel), which showed that Cdk1 protein can physically bind to Oct4 through a direct protein–protein association. It was reported that Cdk1 shares high identity in sequence with other Cdk members, such as Cdk2 and Cdk4. Moreover, their function seems to partially compensate [23]. Therefore, we were interested to investigate whether Cdk2/4 could similarly interact with Oct4. To achieve this, similar experiments with figure 2C were performed by using purified Oct4 and Cdk2/4 protein. Neither Cdk2 nor Cdk4 was able to form complex with Oct4 (Fig. 2D, left and middle panel). We also carried out the Oct4 IP experiment and did not identify a direct physical association between them (Fig. 2D, right panel).

To further explore the interaction between Cdk1 and Oct4 in self-renewing ES cells in vivo, we performed the Immunocytochemistry assay. Importantly, most cells exhibited foci formed by Cdk1 and Oct4 co-localization (Fig. 3).

3.3. Cdk1 enhanced the suppression of Cdx2 transcription by Oct4

It was reported that Oct4 can inhibit the gene activity of *Cdx2* through direct binding to its promoter [11]. Therefore, we asked whether the formation of Cdk1–Oct4 complex can influence binding of Oct4 on the *Cdx2* promoter. To answer this question, electrophoretic mobility shift assay (EMSA) was performed using a biotin-labeled double-stranded oligonucleotide probe, a 40-bp sequence from the *Cdx2* proximal promoter containing the Oct4 binding consensus. Purified GST–Oct4 protein (lane 3), but neither GST nor GST–Cdk1, shifted the *Cdx2* probe, indicating the formation of *Cdx2* probe–Oct4 complex (Fig. 4A). Interestingly, although Cdk1 alone failed in associating with the probe, the bands representing shifted *Cdx2* probe–Oct4 complex was significantly enhanced by addition of purified GST–Cdk1. Moreover, more purified GST–Cdk1 was added, more shifted bands were observed (lane 6–7, Fig. 4A). Meanwhile, compared with the wild-type Cdk1, kinase-dead Cdk1 mutant exhibited similar activity in enhancing Oct4 binding on the *Cdx2* promoter (lanes 8 and 9, Fig. 4A), which was consistent with the capability of Cdk1 mutant in interacting with Oct4 (Fig. 2A). This observation showed that Cdk1 could not directly bind to the *Cdx2* promoter. However, it enhanced the binding of Oct4 on the *Cdx2* gene.

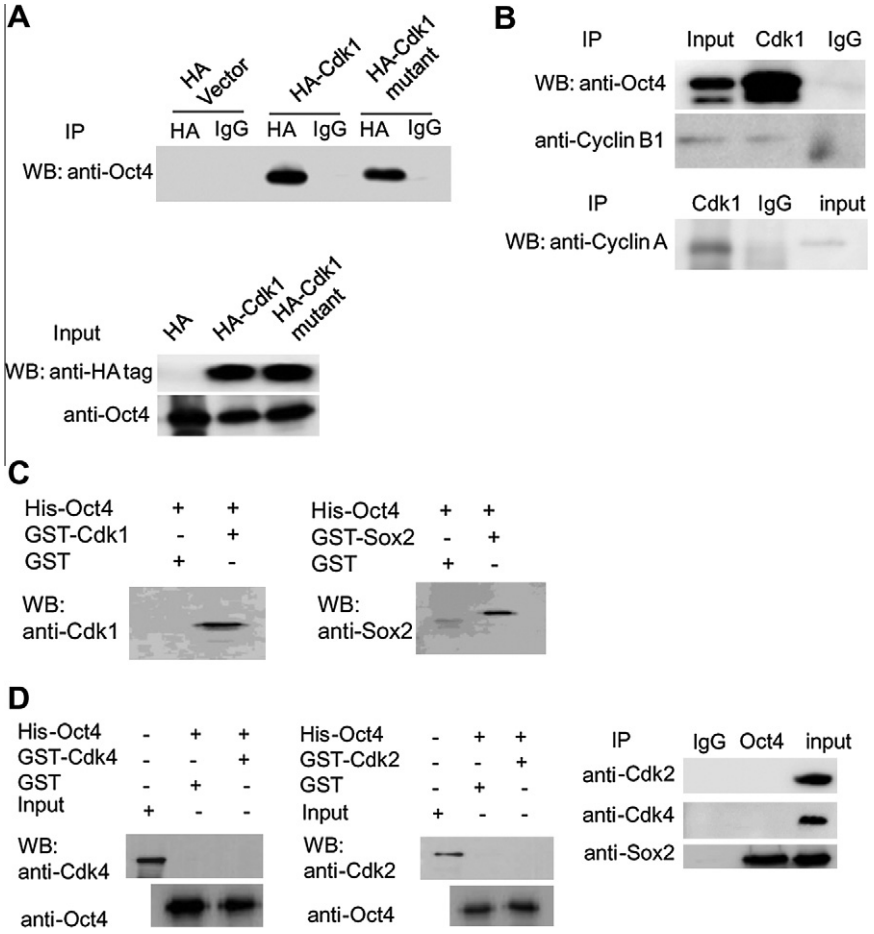

Fig. 2. Cdk1 directly interacted with Oct4. (A) The interaction between Cdk1 and Oct4 was detected in *Cdk1*or *Cdk1* mutant-overexpressed ES cells. ES cells were transfected with pcDNA-HA expression vectors encoding Cdk1 or Cdk1 kinase-dead mutant. Whole cell extracts were prepared 48 h after transfection. To monitor the expression level of exogenous Cdk1 or Cdk1 mutant and endogenous Oct4, the extracts were analyzed by Western blotting with the antibodies against HA and Oct4 respectively (lower panels). The extracts were further immunoprecipitated with anti-HA monoclonal antibody, followed by Western blotting with anti-Oct4 monoclonal antibody (upper panel). (B) The interaction between endogenous Cdk1 and Oct4 was detected in wild type ES cells. Extracts of ES cells were immunoprecipitated with anti-Cdk1 antibody or mouse IgG, followed by Western blotting with the anti-Oct4 antibody. Cyclin A and Cyclin B1 were used as positive controls. (C) In vitro examination of Cdk1–Oct4 complex formation through the pull-down assay. Ni–NTA agarose beads were used to bind His-tagged Oct4 so as to capture GST-tagged Cdk1 purified from bacteria extracts. GST-tagged Sox2 was used as a positive control. (D) Cdk2 and Cdk4 failed to associate with Oct4. Ni–NTA agarose beads were used to bind His-tagged Oct4 so as to capture GST-tagged Cdk2 (middle panel) and Cdk4 (left panel) purified from bacteria extracts and followed by GST-pull down assay. Extracts of ES cells were immunoprecipitated with anti-Oct4 antibody or mouse IgG, followed by Western blotting with the antibodies against Cdk2 and Cdk4 respectively. The antibody against Sox2 was used as a positive control (right panel).

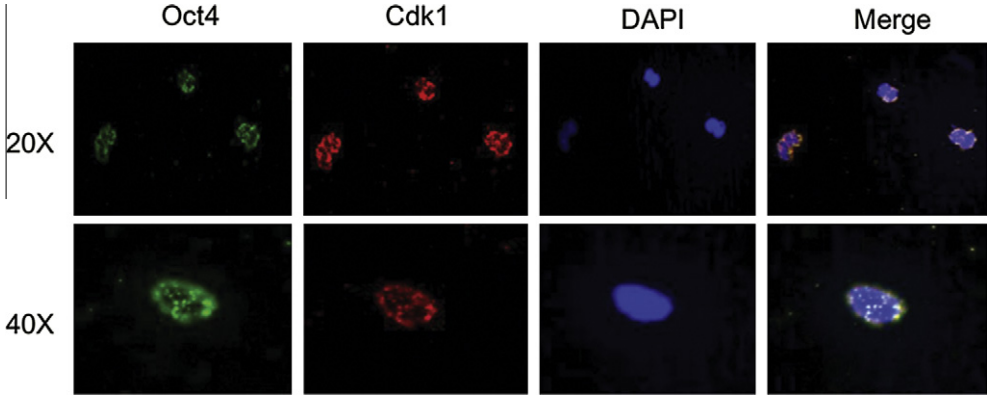

Fig. 3. The immunocytochemistry assay was performed to detect the foci formed by Cdk1 and Oct4 co-localization. The subcellular localization of endogenous Oct4 (green) and Cdk1 (red) was analyzed by immunocytochemistry in ES cells. Cell nuclei were stained with DAPI (40,6-diamidino-2-phenylindole, blue). Images were captured with 20× and 40× immersion objective lenses.

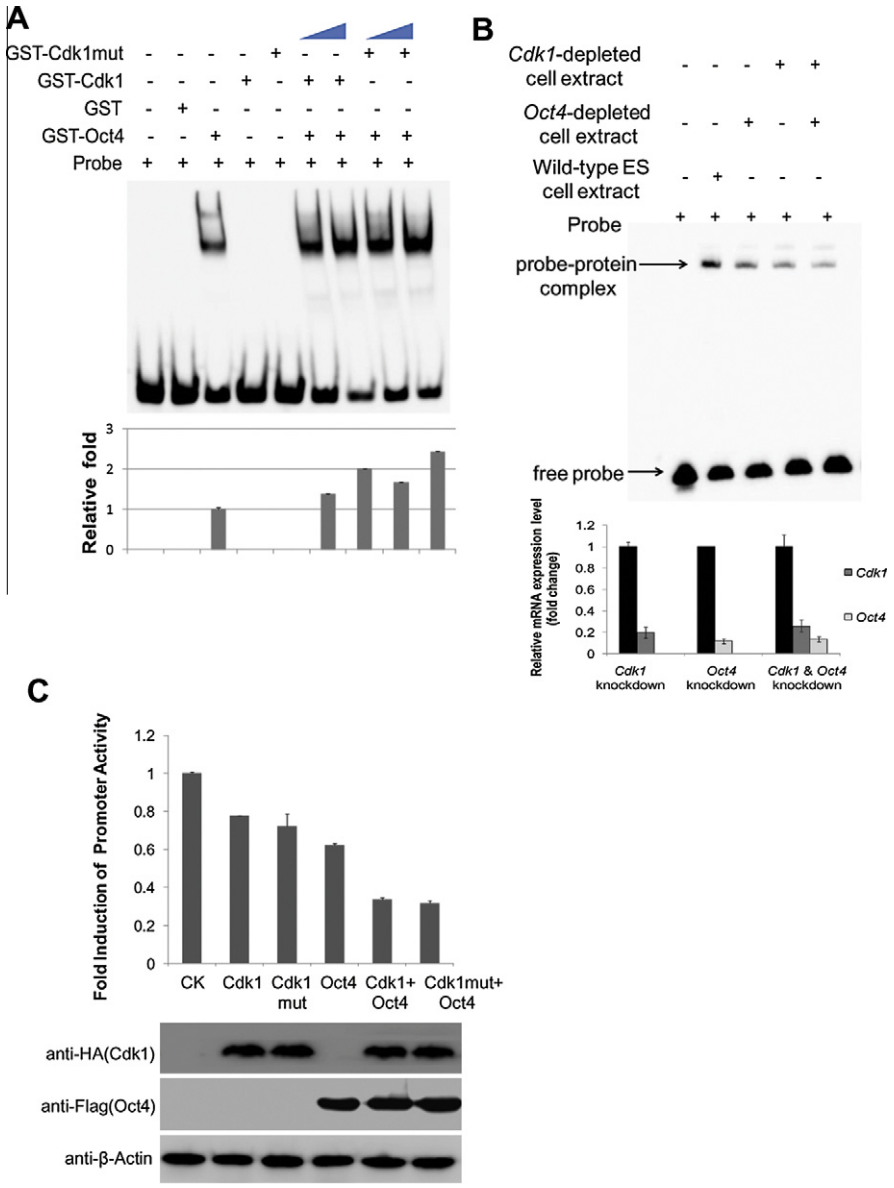

Fig. 4. Cdk1 enhanced the transcription repression of *Cdx2* by interacting with Oct4. (A) Purified GST–Oct4 was incubated with either probe alone or increased amount of purified GST–Cdk1 or GST–Cdk1 mutant (kinase-dead), followed by the EMSA with the probe designed from the *Cdx2* promoter with known Oct4 binding site (upper panel). AlphaEaseFC (Alpha Innotech) software was used to quantify the density of shifted bands formed by protein and probe association (lower panel). (B) Mouse ES cells were transfected with *Cdk1* depletion construct, *Oct4* depletion construct or both. Levels of *Cdk1* or *Oct4* after knockdown were determined by real-time RT-PCR (right panel). *Cdk1*-depleted, *Oct4*-depleted or *Cdk1*/*Oct4* double-depleted cell lysates were incubated with the *Cdx2* probe and followed by the EMSA (left panel). (C) The *Cdx2* promoter–Luciferase reporter was co-transfected to 293 cell line with the constructs over-expressing HA-tagged Cdk1 or Cdk1 mutant together with the one over-expressing Flag-tagged Oct4. Single overexpression of Cdk1, Cdk1 mutant or Oct4 was also used to explore their effect on regulating the *Cdx2* promoter activity. Fourty eight hour after transfection, luciferase activities were determined (upper panel). The data was normalized to the activity of cells transfected with the empty vector (pGL4.2). Western blotting assay was used to monitor the expression levels of HA-tagged Cdk1 and Flag-tagged Oct4 (lower panel).

On the other hand, to further address the biological significance of Cdk1 in the regulation of *Cdx2* expression, we generated the *Cdk1*-depleted, *Oct4*-depleted, or *Cdk1* and *Oct4* double depleted ES cells to further explore how Cdk1 was involved in Oct4-mediated transcription regulation of *Cdx2*. Three days after transfection, whole cell lysates were harvested. The knockdown constructs efficiently reduced endogenous Cdk1 and Oct4 mRNA by about 80% and 90%, respectively (Fig. 4B, right panel). Western blotting analysis further confirmed the successful depletion of these two genes, while Oct4 expression level was not affected upon *Cdk1* knockdown (data not shown; [16]). We further used these cell lysates to perform EMSA. As shown in Fig. 4B, compared with wild type ES cells, both *Cdk1* depletion and *Oct4* depletion resulted in a decrease in Oct4-*Cdx2* probe formation (lanes 3 and 4). Furthermore, knockdown *Cdk1* in the *Oct4*-depleted ES cells led to a further decrease in the formation of probe-protein complex by 82% (lane 5). We concluded that although Cdk1 alone could not associate with *Cdx2*, the interaction between Cdk1 and Oct4 allowed Cdk1 to enhance the binding of Oct4 on *Cdx2* gene.

Our previous study showed that the expression of *Cdx2* was induced significantly upon the knockdown of *Cdk1* [16]. Therefore, the next question of interest was whether *Cdk1* depletion-induced *Cdx2* repression was due to its interaction with Oct4. To answer this question, we carried out luciferase assay with the reporter harboring the *Cdx2* promoter (P_{Cdx2}–Luc). The P_{Cdx2}–Luc was co-transfected to 293 cell line with the constructs overexpressing

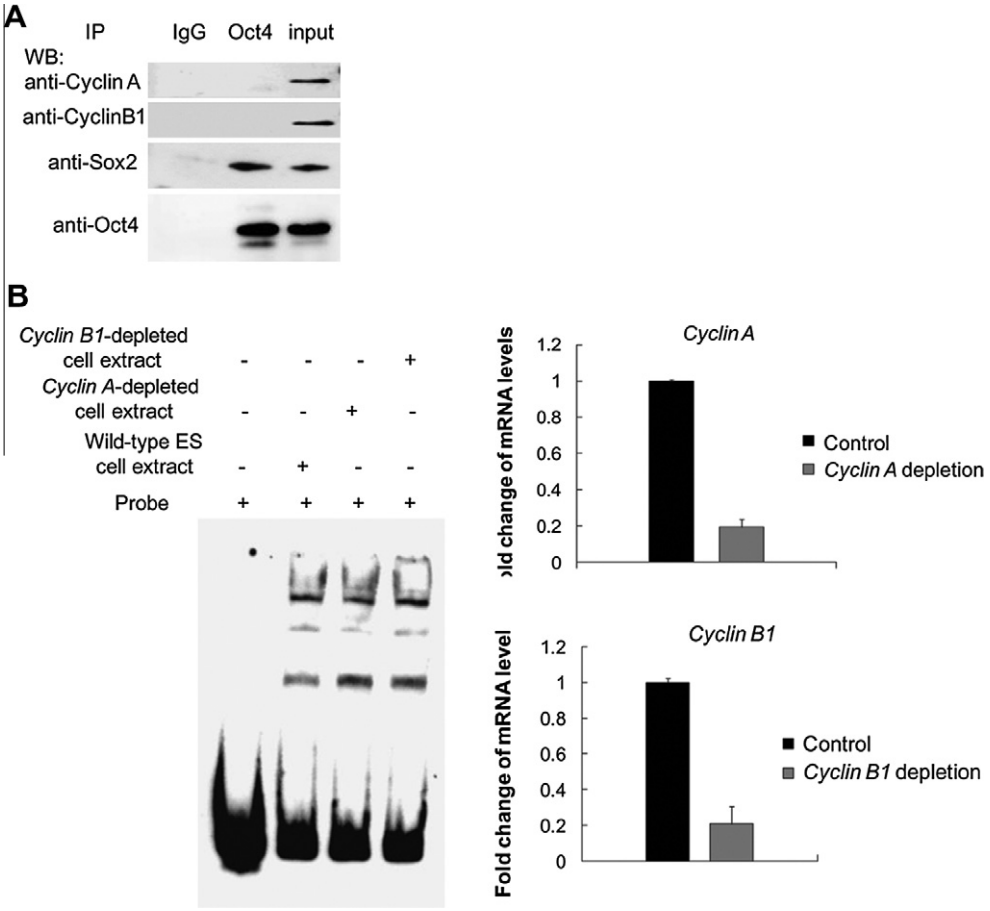

Fig. 5. The role of Cdk1 involved in Oct4-mediated transcription regulating in ES cells was Cyclin-independent. (A) The interaction between endogenous Oct4 with Cyclin A/B was not detected. Extracts of ES cells were immunoprecipitated with anti-Oct4 antibody or mouse IgG, followed by Western blotting with the antibodies against Cyclin A/B. Sox2 was used as a positive control as the known Oct4 partner. (B) Mouse ES cells were transfected with *Cyclin A* depletion construct or *Cyclin B1* depletion construct. Levels of gene expression were determined by real-time RT-PCR (right panel). Cell lysates with depleted *Cyclin A*, *or Cyclin B1* were incubated with the *Cdx2* probe containing Oct4 binding site and followed by an EMSA.

HA-tagged Cdk1 or Cdk1 mutant together with the one overexpressing Flag-tagged Oct4. Single overexpression of Cdk1, Cdk1 mutant or Oct4 was also used to explore their effect on regulating the *Cdx2* promoter activity. Western blotting assay showed a successful overexpression of Cdk1, Cdk1 mutant and Oct4, respectively (Fig. 4C). Upon Cdk1 or Oct4 overexpression, the activity of *Cdx2* promoter dramatically decreased by about 30% and 40%, respectively, and Cdk1 mutant exhibited similar effect with the wild-type Cdk1 on inhibiting the promoter (Fig. 4C). Furthermore, when we overexpressed either Cdk1 or Cdk1 mutant in *Oct4*-overexpressed ES cells, the activity of *Cdx2* promoter was further decreased by about 70% compared with control (Fig. 4C). These results demonstrated that Cdk1–Oct4 complex formation significantly enhanced the repressive role of Oct4 in regulating *Cdx2* promoter activity. Moreover, this regulation was independent on the kinase activity of Cdk1.

The next question we were interested to address was whether the role of Cdk1 in ES cells was cell cycle-autonomous. Cyclin A and Cyclin B1 are known partners of Cdk1 in the process of cell cycle regulation [24]. Thus, we designed experiments to explore whether these two Cyclin proteins held similar role with Cdk1 in regulating Oct4-mediated *Cdx2* repression. Firstly, we performed Oct4 immunoprecipitation experiment and found that neither Cyclin A nor Cyclin B1 was detected in the Oct4 complex (Fig. 5A). Furthermore, we generated *Cyclin A*- or *Cyclin B1*-depleted ES cell extract for the EMSA. Our result showed *Cyclin A* or *Cyclin B* depletion did not change Oct4 binding on *Cdx2* promoter (Fig. 5B).

Combining the results uncovered by the luciferase assay and EMSA experiment, we concluded that the interaction between Cdk1 and Oct4 is required by the Oct4-mediated transcriptional suppression of *Cdx2*. Base on our findings, a model was generated (Fig. 6B). Cdk1 can directly interact with Oct4 to enhance the transcriptional inhibition of Cdx2 by Oct4. As a result, ES cells maintained the propertied and stopped to differentiate to trophectoderm lineage.

4. Discussion

In eukaryotic cells, the cell cycle is controlled by Cdks and their binding partners, Cyclins [13]. In early G1 phase, Cdk4 and/or Cdk6 are activated by D-type Cyclins and initiate phosphorylation of the Retinoblastoma protein (Rb) family and release E2F [24,25]. During the G2/M transition, Cdk1/Cyclin A activity is required for the initiation of prophase [26]. Finally, Cdk1/Cyclin B complex actively participates and completes mitosis [27]. Compared with the normal cells, ES cells have a short cell cycle (around 11 h), primarily owing to a short G1 phase [28]. The Rb protein keeps hyperphosphorylated and maintains inactive throughout the cell cycle, resulting in constitutive E2F activation and subsequent transcription of its target genes. This explains the G1/S checkpoint absence

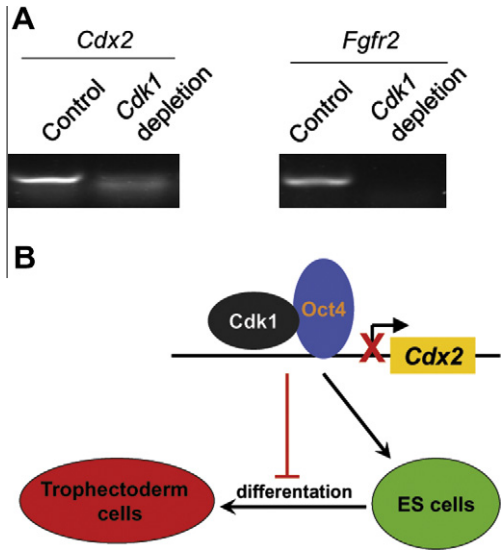

Fig. 6. Cdk1 enhanced Oct4-mediated transcription repression of trophectoderm markers and thus was involved into inhibiting ES cell differentiation into trophectoderm. (A) Chromatin Immunoprecipitation assay was used to analyze the effect of *Cdk1* depletion on Oct4's binding on trophectoderm marker *Cdx2* and *Fgfr2*. *Cdk1* depleted-ES cell Chromatin extract was immunoprecipitated with the Oct4 antibody, and eluted DNA samples were then amplified using primers for the proximal promoter of *Cdx2* or the first intron of *Fgfr2*. Mock depletion sample was used as control. (B) Schematic representation of the interplay between Cdk1 and Oct4 in ES cells. Cdk1 can directly interact with Oct4 to enhance its inhibition of *Cdx2* transcript, consequently maintaining the properties of ES cells while blocking cell differentiation into the trophectoderm lineage.

and active proliferation of ES cells. Among the cell cycle regulators, Cdk1 is highly overexpressed in pluripotent stem cells when compared with somatic cells [29]. This indicates the involvement of Cdk1 in regulating cell differentiation, which has been confirmed by recent studies. Ullah et al. reported that RO3306-mediated Cdk1 inhibition stops cell cycle transition into mitosis, and thus induced differentiation of trophectoderm stem cells into giant cells [15]. Zhang et al. used RNA interference assay to demonstrate that *Cdk1* depletion leads to ES cells arrest at G2 phase and consequently apoptosis [16]. Consistently, *Cdk1* deletion leads to early embryonic lethality [14]. Although the *Cdk1*-depleted ES cells have been characterized, how Cdk1 maintains the normal self-renewing undifferentiated status of the ES cells remained unclear. This study demonstrated the direct interaction between Cdk1 and Oct4. More importantly, transcriptional repression of *Cdx2* by Oct4 was dependent on the Cdk1–Oct4 complex formation. This finding is consistent with previous observation that *Cdk1*-depleted ES cells exhibit a dramatic increase in Cdx2 transcript [16].

As shown by our previous study, Cdx2 is one of the highlighted genes whose expression shows significant changed [16]. For example, Mesoendoderm marker *Msx1* and ectoderm *Fgf5* are also greatly up-regulated upon *Cdk1* depletion. The reason for us to focus on *Cdx2* is that it is a well established target of Oct4 although genome-wide chromatin-immunoprecipitation (ChIP)-sequencing results have shown *Msx1* and *Fgf5* among the Oct4 target gene list [30]. On the other hand, Oct4 is the key inhibitor to stop ES cells differentiating into Cdx2-marked trophectoderm [6]. As a protein–protein interaction partner of Oct4, the induction of *Cdx2* upon *Cdk1* depletion supports the possibility that Cdk1 and Oct4 function in repressing trophectoderm differentiation through the Cdk1–Oct4 complex formation. To confirm this, we further performed in vivo ChIP assay with *Cdk1*-depleted ES cells and found that *Cdk1* depletion significantly reduced Oct4's binding on *Cdx2* and another trophectoderm marker, *Fgfr2* (Fig. 6A). Besides differentiation marker genes, a list of self-renewal related genes, such as *Sox2*, *Esrrb*, *Tdgf1* and *Tcl1*, show down-regulated in *Cdk1* knockdown ES cells [16]. Of note, these genes are binding targets of Oct4 [30]. Thus, whether Cdk1 is involved in promoting Oct4's activation of these genes remains to be answered.

Different post-translational modifications of Oct4 have been identified. For example, E3 ubiquitin ligase Wwp2 can mediate ubquitination of Oct4 to enhance its instability in ES cells [31]. Potential protein kinase A (PKA) phosphorylates serine 229 of Oct4 [32]. Other phosphorylation sites of Oct4 were also identified [33]. These modifications may serve to influence Oct4 homo- or heterodimer formation, consequently influencing its transcriptional regulation of downstream targets. Although Cdk1 is a well-known kinase required by the procession of eukaryotic cell cycle, the direct association of Oct4 with Cdk1 may not promise an effective phosphorylation. Several findings from this study can be as evidences. Firstly, Cdk1 interacted with Oct4 in a kinase-independent manner. And kinase-dead mutation in Cdk1 did not influence its interplay with Oct4 in regulating *Cdx2* transcription. Secondly, neither Cyclin A nor Cyclin B1 was associated with the Oct4 interactome. Moreover, these two factors did not involve in Oct4-mediated *Cdx2* repression. Thus, we presented a novel role of Cdk1 with kinase- and Cyclin-independence in ES cells. Further investigation along this line will be of great interest to the field.

In conclusion, our study confirmed the direct interaction of Cdk1 and Oct4. Moreover, this interaction promoted the binding of Oct4 on the *Cdx2* promoter and enhanced Oct4's repression on *Cdx2* gene activity. These findings enriched our understanding of how Cdk1 collaborates with transcription factor Oct4 to inhibit differentiation of ES cells into trophectoderm, and thus maintains the undifferentiated state of ES cells.

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